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Abstract: Sialyl Lewis x (sLex) is an established selectin ligand occurring on N- and O-linked glycans. Using a completely enzymic approach starting from p-nitrophenyl N-acetyl- β -D-galactosaminide (GalNAc(1-pNp) as core substrate, the sLex-oligosaccharide Neu5Ac(2-3)Gal(1-4)[Fuc(1-3)]GlcNAc(1-6)[Gal(1-3)]GalNAc(1-pNp, representing the O-linked form, was synthesized in an overall yield of 32%. In a first step, Gal(1-3)GalNAc(1-pNp was prepared in a yield of 52% using UDP-Gal and an enriched preparation of β -galactosyltransferase (EC 2.4.1.122) from rat liver. UDP-GlcNAc and a recombinant affinity-purified preparation of core 2 β -N-acetylglucosaminyltransferase (EC 2.4.1.102) fused to Protein A were used to branch the core 1 structure, affording GlcNAc(1-6)[Gal(1-3)]GalNAc(1-pNp in a yield of >85%. The core 2 structure was galactosylated using UDP-Gal and purified human milk β -galactosyltransferase 1 (EC 2.4.1.38) (yield of >85%), then sialylated using CMP-Neu5Ac and purified recombinant β -sialyltransferase 3 (EC 2.4.99.X) (yield of 87%), and finally fucosylated using GDP-Fuc and recombinant human β -fucosyltransferase 6 (EC 2.4.1.152) produced in *Pichia pastoris* (yield of 100%). Overall 1.5 μ mol of product was prepared. MALDI TOF mass spectra, and 1D and 2D TOCSY and ROESY 1 H NMR analysis confirmed the obtained structure

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Complete enzymic synthesis of the mucin-type sialyl Lewis x epitope, involved in the interaction between PSGL-1 and P-selectin

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Sialyl Lewis x (sLe^x) is an established selectin ligand occurring on N- and O-linked glycans. Using a completely enzymic approach starting from *p*-nitrophenyl *N*-acetyl- α -D-galactosaminide (GalNAc(α 1-pNp) as core substrate, the sLe^x-oligosaccharide Neu5Ac(α 2-3)Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp), representing the O-linked form, was synthesized in an overall yield of 32%. In a first step, Gal(β 1-3)GalNAc(α 1-pNp) was prepared in a yield of 52% using UDP-Gal and an enriched preparation of β 3-galactosyltransferase (EC 2.4.1.122) from rat liver. UDP-GlcNAc and a recombinant affinity-purified preparation of core 2 β 6-*N*-acetylglucosaminyltransferase (EC 2.4.1.102) fused to Protein A were used to branch the core 1 structure, affording GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp) in a yield of >85%. The core 2 structure was galactosylated using UDP-Gal and purified human milk β 4-galactosyltransferase 1 (EC 2.4.1.38) (yield of >85%), then sialylated using CMP-Neu5Ac and purified recombinant α 3-sialyltransferase 3 (EC 2.4.99.X) (yield of 87%), and finally fucosylated using GDP-Fuc and recombinant human α 3-fucosyltransferase 6 (EC 2.4.1.152) produced in *Pichia pastoris* (yield of 100%). Overall 1.5 μ mol of product was prepared. MALDI TOF mass spectra, and 1D and 2D TOCSY and ROESY ¹H NMR analysis confirmed the obtained structure.

Keywords: sialyl Lewis x, enzymic synthesis, *N*-acetylglucosaminyltransferase, fucosyltransferase, galactosyltransferase, sialyltransferase, recombinant glycosyltransferases, P-selectin, PSGL-1

Abbreviations: β 3-GalT, UDP-Gal:GalNAc α -R β -1,3-galactosyltransferase, β 3-galactosyltransferase; C2GnT, UDP-GlcNAc:Gal(β 1-3)GalNAc(α 1-R) (GlcNAc to GalNAc) β -1,6-*N*-acetylglucosaminyltransferase, β 6-*N*-acetylglucosaminyltransferase; β 4-GalT1, UDP-Gal:GlcNAc β -1,4-galactosyltransferase 1, β 4-galactosyltransferase 1; ST3Gal3, CMP-Neu5Ac:Gal(β 1-4)GlcNAc α -2,3-sialyltransferase 3, α 3-sialyltransferase 3; α 3-FucT6, GDP-Fuc:Gal(β 1-4)GlcNAc (Fuc to GlcNAc) α -1,3-fucosyltransferase 6, α 3-fucosyltransferase 6; Caco, sodium cacodylate (Na(CH₃)₂AsO₂) - HCl buffer; CIAP, Calf Intestinal Alkaline Phosphatase; core 1, Gal(β 1-3)GalNAc; core 2, GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc; core 4, GlcNAc(β 1-6)[GlcNAc(β 1-3)]GalNAc; core 6, GlcNAc(β 1-6)GalNAc; FPLC, Fast Protein Liquid Chromatography; HPLC, High Performance Liquid Chromatography; LacNAc, Gal(β 1-4)GlcNAc; lacto-*N*-biose, Gal(β 1-3)GlcNAc; MALDI TOF, Matrix Assisted Laser Desorption Ionisation Time of Flight; MES, 2-(*N*-Morpholino)ethanesulfonic acid - NaOH buffer; Me₂SO, Dimethyl sulfoxide; MLEV, Malcolm Levitt; NMR, Nuclear Magnetic Resonance; pNp, *p*-Nitrophenyl; PSGL-1, P-Selectin Glycoprotein Ligand 1; ROESY, Rotating Frame Nuclear Overhauser Enhancement Spectroscopy; sLe^x, Sialyl Lewis x; TOCSY, Total Correlation Spectroscopy; WEFT, Water Eliminated Fourier Transform.

Introduction

Selectin-mediated cell adhesion via the sialyl Lewis x (sLe^x) epitope (Neu5Ac(α 2-3)Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-)

[1,2] is playing an important role in many pathophysiological processes, e.g. in cancer [3,4], inflammatory diseases (reviewed in [5,6]), and acute rejections of solid organ transplants [7]. Oligosaccharides containing the sLe^x structure have been demonstrated *in vivo* [8] and *in vitro* [9] to inhibit E-, L- and P-selectin-mediated adhesive interactions. Up till now these components as well as their mimetics have been tested and demonstrated to be beneficial in lung injury [10], myocardial ischemia and reperfusion injury [11,12] as well as in the inhibition of angiogenesis [13].

The description of a similar synthesis was published by Leppanen et al. (*J Biol Chem* 1999 Aug 27;274(35):24838–48 after this paper was submitted (Aug. 2, 1999).

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Blocking sLe^x-selectin binding, therefore, represents a highly relevant therapeutic target. To develop selectin blockers, different strategies have been followed. Since the first chemical synthesis of sLe^x in 1991 [14], several elegant organic synthetic routes for this epitope have been described [14–16]. Alternatively, enzymic as well as chemo-enzymic approaches using glycosyltransferases and/or glycosidases have been investigated [17,18]. The quite low affinity of simple sLe^x oligosaccharides [19–21] as well as the short life time in the circulation both led to the development of multiple sLe^x-containing structures [22,23] and numerous mimetics (reviewed in [24]), showing enhanced binding properties compared to simple sLe^x [25,26]. While the analogues may be toxic or antigenic, the synthesis of complex glycoconjugates based on naturally occurring selectin ligands seems to be a possible alternative. A promising candidate is the hexasaccharide O-linked to Thr57 of the N-terminus of PSGL-1, one of the best characterized glycoproteins involved in P- and E-selectin binding [27].

Here, we report the completely enzymic synthesis of O-linked core 2 type sLe^x, Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)[Gal(β1-3)]GalNAc(α1-pNp, based on the sequential transfer of appropriate monosaccharides from nucleotide donors using suitable glycosyltransferases as biocatalysts. The identify of this product is confirmed by MALDI TOF mass spectrometry, 1D and 2D TOCSY and ROESY ¹H NMR analysis.

Materials and methods

Chemicals

All reagents were commercially available and of highest purity unless otherwise noted. UDP-Gal, UDP-GlcNAc, CMP-Neu5Ac, GDP-Fuc, and GalNAc(α1-pNp were purchased from Sigma (Buchs, Switzerland). The corresponding ¹⁴C-labeled nucleotide sugars were obtained from Amersham International plc (Zürich, Switzerland). ²H₂O was purchased from Isotec (Veenendaal, The Netherlands), HPLC-grade acetonitrile from Rathburn (Walkerburn, Scotland), and ammonium bicarbonate and 6-aza-2-thiothymine from Sigma (Zwijndrecht, The Netherlands). Scintillant Irga-Safe Plus was purchased from Packard (Zürich, Switzerland), and calf intestinal alkaline phosphatase (CIAP) from Boehringer Mannheim (Mannheim, Germany).

Preparation of glycosyltransferases

β3-Galactosyltransferase (β3-GalT). Following the methods of Schachter and Brockhausen [28,29], 40 g of fresh rat liver taken from 7 month old Long evans male were rinsed, minced with scissors and homogenized in 80 ml of 50 mM MES, pH 6.5, containing 250 mM sucrose and 20 mM MgCl₂, in a 1 l waring blender (two 20 s burst at high settings, with 40 s rest). After centrifugation at 680 g for 10

min, the pellet was homogenized using a Potter-Elvehjem glass homogenizer with a motor driven pestle by making three passes at 800 rpm. Then, the homogenate was centrifuged for 1 h at 10,000 g, and 22.5 ml of the pellet were extracted five times overnight with intermediate centrifugation at 100,000 g using equal volumes of 50 mM MES, pH 6.5, containing 5 mM MnCl₂, 0.02% NaN₃, 1% Triton X-100, and 0.1 M NaCl. The highest activity was found in the third extract after 3 days of incubation. All steps were performed at 4 °C.

α3-Fucosyltransferase 6 (α3-FucT6). Human α3-FucT6 (GenBank Accession number M98825) was produced using the *Pichia pastoris* expression system from Invitrogen (Groningen, The Netherlands). The generation of the *Pichia* strain will be described elsewhere. Briefly, a strain was used that secreted 1 U/l of a soluble form of α3-FucT6 into the supernatant, as measured by using *N*-acetylglucosamine (LacNAc) as acceptor. The enzyme was enriched by ultrafiltration and affinity-purified on GDP-hexanamine-agarose.

Other glycosyltransferases. Recombinant mouse core 2 β6-*N*-acetylglucosaminyltransferase (C2GnT) (GenBank Accession number U19265) was expressed as a Protein A tagged soluble enzyme in CHO cells and purified as described previously [30]. β4-Galactosyltransferase 1 (β4-GalT1) was purified from human milk as described previously [31]. Purified recombinant rat α3-sialyltransferase 3 (ST3Gal3) (GenBank Accession number M97754), produced in Sf9 cells, was obtained as a gift from M. Streiff (Novartis Pharma, Basel, Switzerland).

Enzymic synthesis protocols

The experimental conditions for the various steps in the enzymic synthesis of Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-6)[Gal(β1-3)]GalNAc(α1-pNp are presented in Table 1. All incubations of saccharide acceptors and nucleotide sugar donors in the presence of glycosyltransferases were carried out at 37 °C in a water bath, except for the incubation related to β3-GalT (25 °C).

Yields were determined via parallel incubations with ¹⁴C-labeled nucleotide sugars, using 6–13% of the incubation mixtures; 20-μl samples of these mixtures were taken at different time intervals. Reactions were stopped by adding 0.5 ml of ice-cold water. Separations were carried out on a Sep-Pak C₁₈ cartridge (Waters, Milford, USA) mounted on a vacuum chamber, equilibrated with 10 ml of MeOH followed by 10 ml of H₂O. To remove unreacted radiolabeled nucleotide sugar, the cartridge was washed with 15 ml of H₂O. The non-radioactive acceptor and the radiolabeled product were eluted with 5 ml of MeOH, and the eluate was mixed with 10 ml of scintillant Irga-Safe Plus. Typically, cpm values of 1,000–5,000 were counted.

The non-radioactive incubation mixtures were separated as mentioned above, using three sequentially coupled Sep-

Table 1. Reaction parameters used for the sequential build-up of the core 2 type sLe^x epitope

Step	Acceptor	Donor	Transferase	Temperature (°C)	Other Components	Time (h)	Step yield
1a	15 mM (=10.3 mg) GalNAc(α1-pNp)	20 mM (=24.4 mg) UDP-Gal	enriched rat liver β3-GalT (5.6 mU)	25	1% Triton X-100 10% Me ₂ SO 0.1 M MES pH 6.5 10 mM MnCl ₂ 60 U CIAP	72	29%*
1b	10 mM (=10.3 mg) GalNAc(α1-pNp)	20 mM (=24.4 mg) UDP-Gal	enriched rat liver β3-GalT (3 mU)	25	1% Triton X-100 10% Me ₂ SO 0.1 M MES pH 6.5 10 mM MnCl ₂ 60 U CIAP	72	23%* 52% total
2	20 mM (=7.8 mg) Gal(β1-3)GalNAc(α1-pNp)	25 mM (=12.6 mg) UDP-GlcNAc	recombinant mouse C2GnT (64 mU)	37	0.1 M MES pH 6.5 10 mM MgCl ₂	32	85%*
3	10 mM (=8.7 mg) GlcNAc(β1-6)[Gal (β1-3)]GalNAc(α1-pNp)	15 mM (=11.3 mg) UDP-Gal	human milk β4-GalT1 (200 mU)	37	0.1 M Tris-HCl pH 7.4 10 mM MgCl ₂	5	85%*
4a	10 mM (=8.3 mg) Gal(β1-4)GlcNAc(β1-6)[Gal (β1-3)]GalNAc(α1-pNp)	15 mM (=8.8 mg) CMP-Neu5Ac	recombinant human ST3Gal3 (170 mU)	37	0.1 M Caco pH 6.8 10 mM MgCl ₂ 2 mM CaCl ₂ 20 U CIAP	16	27%*
4b	10 mM (=7.7 mg) Gal(β1-4)GlcNAc(β1-6)[Gal (β1-3)]GalNAc(α1-pNp)	15 mM (=8 mg) CMP-Neu5Ac	recombinant human ST3Gal3 (72 mU)	37	0.1 M Caco pH 6.8 10 mM MgCl ₂ 2 mM CaCl ₂ 20 U CIAP	5	60%* 87% total**
5	3.5 mM (=2 mg) Neu5Ac(α2-3)Gal(β1-4) GlcNAc(β1-6)[Gal (β1-3)]GalNAc(α1-pNp)	20 mM (=6 mg) GDP-Fuc	recombinant human α3-FucT6 (70 mU)	37	25 mM Caco pH 6.2 5 mM ATP 10 mM Fuc 10 mM MnCl ₂ 20 U CIAP	6	100%**

*Calculated by radioactive labeled parallel incubations. Yields are given on the basis of acceptor used for the specific reaction. **Calculated from the HPLC profile.

Pak C₁₈ cartridges. In each case, the MeOH phase was concentrated in a Speed vac, and the residues were used directly in the next glycosylation step, except prior to the fucosylation.

After the sialylation and fucosylation steps, the products were purified via gel filtration and HPLC. Reaction mixtures were desalted on Sephadex G-25 (FPLC system; HiTrap, 4 × 5 ml bedvolume; Pharmacia, Uppsala, Sweden) using 5 mM ammonium hydrogen carbonate as eluent at a flow rate of 1.5 ml/min, and subsequent lyophilization. Effluents were monitored by UV at 214 nm, and conductivity. Sephadex G-25 fractions were further purified by HPLC on a ChromSpher 5 C8 reversed phase column (10 × 250 mm, Chrompack, Bergen op Zoom, The Netherlands) at a flow rate of 2.0 ml/min using a Kratos SF 400 HPLC system (ABI Analytical, Kratos Division). The column was eluted isocratically with solvent A (aqueous 80% acetonitrile) during 5 min, followed by a gradient from 100% solvent A - 0% solvent B (aqueous 20% acetonitrile) to 65% solvent A - 35% solvent B in 18 min. The effluents were monitored at 280 nm using a 757 absorbance detector (ABI Analytical,

Kratos Division). The collected fractions were immediately lyophilized for further analysis.

Mass Spectrometry

Negative-ion mode MALDI TOF mass spectrometric analysis of the products was performed on a Voyager-DE (PerSeptive Biosystems) instrument operating at an accelerating voltage of 22 kV (grid voltage 92%, ion guide wire voltage 0.1%) and equipped with a VSL-337ND-N₂ laser. The samples were dissolved in bidistilled water (1 µg/µl) and subsequently mixed in the sample well with 6-aza-2-thiothymine (10 mg/ml in water:acetonitrile 1:1, v/v) at a ratio of 1:3. Linear mass scans were recorded over 1000 Dalton using a pulse delay time of 90 ns. Recorded data were processed using GRAMS/386 software (v. 3.04, Galactic Industries Corporation).

NMR Spectroscopy

Prior to analysis the reaction products were repeatedly exchanged in ²H₂O (99.9 atom % ²H) with intermediate

lyophilization and finally dissolved in 450 μl $^2\text{H}_2\text{O}$ (99.96 atom % ^2H). Resolution-enhanced ^1H 1D and 2D NMR spectra were recorded on Bruker AMX-500, DRX-500 or DRX-600 (Department of NMR Spectroscopy, Utrecht University) spectrometers, at probe temperatures of 300 K. Chemical shifts (δ) were expressed in parts per million relative to internal acetate (δ 1.908; acetone δ 2.225). HO^2H signal suppression was achieved by applying a WEFT pulse sequence [32] in 1D ^1H experiments and by presaturation for 1 s in 2D experiments. 2D TOCSY spectra were recorded by using MLEV-17 mixing sequences [33] with effective spin-lock times between 20 and 100 ms. 2D ROESY [34] spectra were recorded with a mixing time of 250 ms. The spin-lock field strength corresponded to a 90° pulse of about 120 μs . A 2D 600 MHz off-resonance ROESY spectrum of the final product was recorded according to [35]. The spin-lock field strength corresponded to a 90° pulse of about 115 μs . ^1H 1D and 2D spectra were processed on Silicon Graphics IRIS work stations (Indigo 2 and O2) using XINSP2 software (Bijvoet Center, Department of Bio-Organic Chemistry).

Results

Enzymic synthesis of the mucin-type sialyl Lewis x epitope

A survey of the strategy and the amounts of donors, acceptors, and glycosyltransferases used to build up the O-linked hexasaccharide, Neu5Ac(α 2-3)Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6) [Gal(β 1-3)]GalNAc(α 1-pNp), bearing the sLe^x epitope, are presented in Table 1. Yields were determined via parallel incubations with radioactive nucleotide sugar donors (see Materials and methods). During preliminary studies, the individual glycosyltransfer reactions (except for the sialylation) were optimized, and the products Gal(β 1-3)GalNAc(α 1-pNp) (**1**), Gal(β 1-4)GlcNAc(β 1-6) [Gal(β 1-3)]GalNAc(α 1-pNp) (**2**), and Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp) (**3**) were purified for characterization by mass spectrometry and ^1H NMR spectroscopy (Table 2). Therefore, in the complete enzymic synthesis, as described below, the steps 1–4 were carried out without intermediate chromatographic purifications and detailed structural analysis.

GalNAc(α 1-pNp) was chosen as a starting core substrate. This compound was elongated to yield Gal(β 1-3)GalNAc(α 1-pNp) by using UDP-Gal and a rat liver β 3-GalT (EC 2.4.1.122; 0.5 U/g) Triton X-100 extract. To increase the yield, calf intestinal alkaline phosphatase (EC 3.1.3.1; 2500 U/mg) was added [36]. Even though the activity at 25 $^\circ\text{C}$ was only 67% compared to an incubation at 37 $^\circ\text{C}$, 25 $^\circ\text{C}$ was chosen because of a better stability of the transferase. Furthermore, it was found that at least 17% of UDP-Gal was degraded at 25 $^\circ\text{C}$ within 24 h. For this reason, after 3 days of incubation (step 1a) fresh UDP-Gal and enzyme

preparation were added for another 3 days of incubation (step 1b). In step 1a the product yield was 29%; via step 1b an overall yield of 52% was reached.

For the branching of the core 1 structure Gal(β 1-3)GalNAc(α 1-pNp), yielding the core 2 structure, UDP-GlcNAc and purified mouse recombinant C2GnT (EC 2.4.1.102) were applied (step 2). Mouse C2GnT was expressed as a Protein A fusion in CHO cells [30]. GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp) was obtained in a yield of 85% after 32 h of incubation.

The core 2 structure was further elongated at O4 of GlcNAc using UDP-Gal and purified human milk β 4-GalT1 (step 3). Under the applied conditions Gal(β 1-4)GlcNAc(β 1-6) [Gal(β 1-3)]GalNAc(α 1-pNp) was generated in a yield of >85% after 5 h of incubation.

To sialylate the tetrasaccharide, CMP-Neu5Ac and purified recombinant rat ST3Gal3 (EC 2.4.99.X) were used [37]. In two sequential incubations of 16 h and 5 h (step 4; for conditions, see Table 1) Neu5Ac(α 2-3)Gal(β 1-4)GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp) (**4**) was synthesized in a total yield of 87%. For the purification use was made of HiTrap chromatography and reversed phase HPLC.







Finally, the pentasaccharide (**4**) was fucosylated with GDP-Fuc in the presence of recombinant enriched human α 3-FucT6 (EC 2.4.1.152) (step 5), affording the sLe^x epitope-containing title compound **5** after 6 h of incubation in a yield of 100%, as observed by HPLC and supported by a radioactive parallel incubation. In contrast to insect cell supernatant [38] no exoglycosidase degrading the acceptor substrate for α 3-FucT6 could be detected.






Taken together, 1.5 μmol of the mucin-type sLe^x-containing hexasaccharide could be prepared in an overall yield of 32%, whereby the individual yield of each step is calculated on the basis of the acceptor used.

Structural analysis of synthesized products 4 and 5

The negative-ion mode MALDI TOF mass spectrum (Figure 1a) of compound **4** showed one intense signal at m/z 1159.4, corresponding to the deprotonated pseudo-molecular ion of a pentasaccharide with the bruttoformula Neu5AcHex₂HexNAc₂-pNp (calculated mass 1159.4)

The 1D ^1H NMR spectrum (Figure 2a) of compound **4** revealed three main resonances downfield of the HO^2H signal (δ 4.766) at δ 8.307 ($^3J_{\text{m},\text{o}}$ 9.0 Hz), 7.269 ($^3J_{\text{o},\text{m}}$ 9.0 Hz) and 5.805 ($^3J_{1,2}$ 4.0 Hz) (Table 2). The two most downfield signals were assigned to the *m*- and *o*-protons of the *p*-nitrophenyl aglycon [39], respectively, while the remaining resonance was attributed to the anomeric proton of GalNAc (A; pyranose ring form), α -glycosidically linked to the *p*-nitrophenyl aglycon. Upfield of the HO^2H resonance three additional anomeric resonances at δ 4.538 ($^3J_{1,2}$ 7.5 Hz), 4.467 ($^3J_{1,2}$ 7.5 Hz) and 4.447 ($^3J_{1,2}$ 7.5 Hz) were identified and assigned to β -1,3-linked Gal³ (B; pyranose ring form), β -1,6-linked GlcNAc (C; pyranose ring form), and

Table 2. 500 and 600-MHz ¹H-NMR chemical shifts of pNp-oligosaccharides recorded at 300K referenced to internal acetate δ 1.908 (acetone δ 2.225). Compounds are represented by symbolic shorthand notation , GalNAc; , GlcNAc; , Gal4; , Gal3; , Fuc; and , Neu5Ac.

Residue	Reporter group	1	2	3	4	5
		 -pNp	 -pNp	 -pNp	 -pNp	 -pNp
pNp	C ₆ H ₄ '	8.272(9.0)	8.306(9.0)	8.304(9.0)	8.307(9.0)	8.310(9.0)
	C ₆ H ₄	7.281(9.0)	7.270(9.0)	7.265(9.0)	7.269(9.0)	7.270(9.0)
GalNAc	H-1	5.831(4.0)	5.810(4.0)	5.805(4.0)	5.805(4.0)	5.807(4.0)
	H-2	4.580	4.568	4.565	4.564	4.566
	H-3	4.293	4.294	4.293	4.291	4.291
	H-4	4.316	4.305	4.304	4.303	4.303
	H-5	4.023	4.182	4.167	4.187	4.187
	H-6a	3.738	3.771	3.730	3.776	3.763
	H-6b	3.762	3.999	3.998	3.989	3.994
	CH ₃	2.013	2.004	2.002	2.003	2.006
GlcNAc	H-1	-	4.472(7.5)	4.485(7.5)	4.467(7.5)	4.482(7.5)
	H-2	-	3.593	3.765	3.583	3.764
	H-3	-	3.498	3.765	3.487	3.764
	H-4	-	3.664	3.761	3.663	3.745
	H-5	-	3.503	3.512	3.588	3.512
	H-6a	-	3.595	3.701	n.d.	3.563
	H-6b	-	3.904	3.917	3.971	n.d.
	CH ₃	-	1.901	1.886	1.899	1.890
Gal ⁴	H-1	-	4.386(7.5)	4.370(7.5)	4.447(7.5)	4.428(7.5)
	H-2	-	3.512	3.468	3.540	3.504
	H-3	-	3.650	3.629	4.097	4.096
	H-4	-	3.918	3.889	3.951	3.935
	H-5	-	n.d.	3.697	n.d.	3.717
Gal ³	H-1	4.548(7.5)	5.541(7.5)	4.539(7.5)	4.538(7.5)	4.545(7.5)
	H-2	3.560	3.555	3.549	3.553	3.555
	H-3	3.657	3.654	3.639	3.654	3.646
	H-4	3.932	3.933	3.927	3.928	3.937
	H-5	n.d.	n.d.	3.652	n.d.	3.704
	H-6a	n.d.	n.d.	3.857	n.d.	3.881
	H-6b	n.d.	n.d.	3.765	n.d.	3.740
Fuc	H-1	-	-	5.034(4.0)	-	5.030(4.0)
	H-2	-	-	3.651	-	3.645
	H-3	-	-	3.771	-	3.763
	H-4	-	-	3.849	-	3.849
	H-5	-	-	4.776	-	4.777
	CH ₃	-	-	1.151(6.5)	-	1.147(6.5)
Neu5Ac	H-3a	-	-	-	1.790	1.789
	H-3e	-	-	-	2.753	2.763
	H-4	-	-	-	3.670	3.667
	H-5	-	-	-	3.834	3.848
	H-6	-	-	-	3.636	3.636
	H-7	-	-	-	n.d.	3.605
	H-8	-	-	-	n.d.	3.889
	H-9a	-	-	-	n.d.	3.865
	H-9b	-	-	-	n.d.	3.649
	CH ₃	-	-	-	2.030	2.036

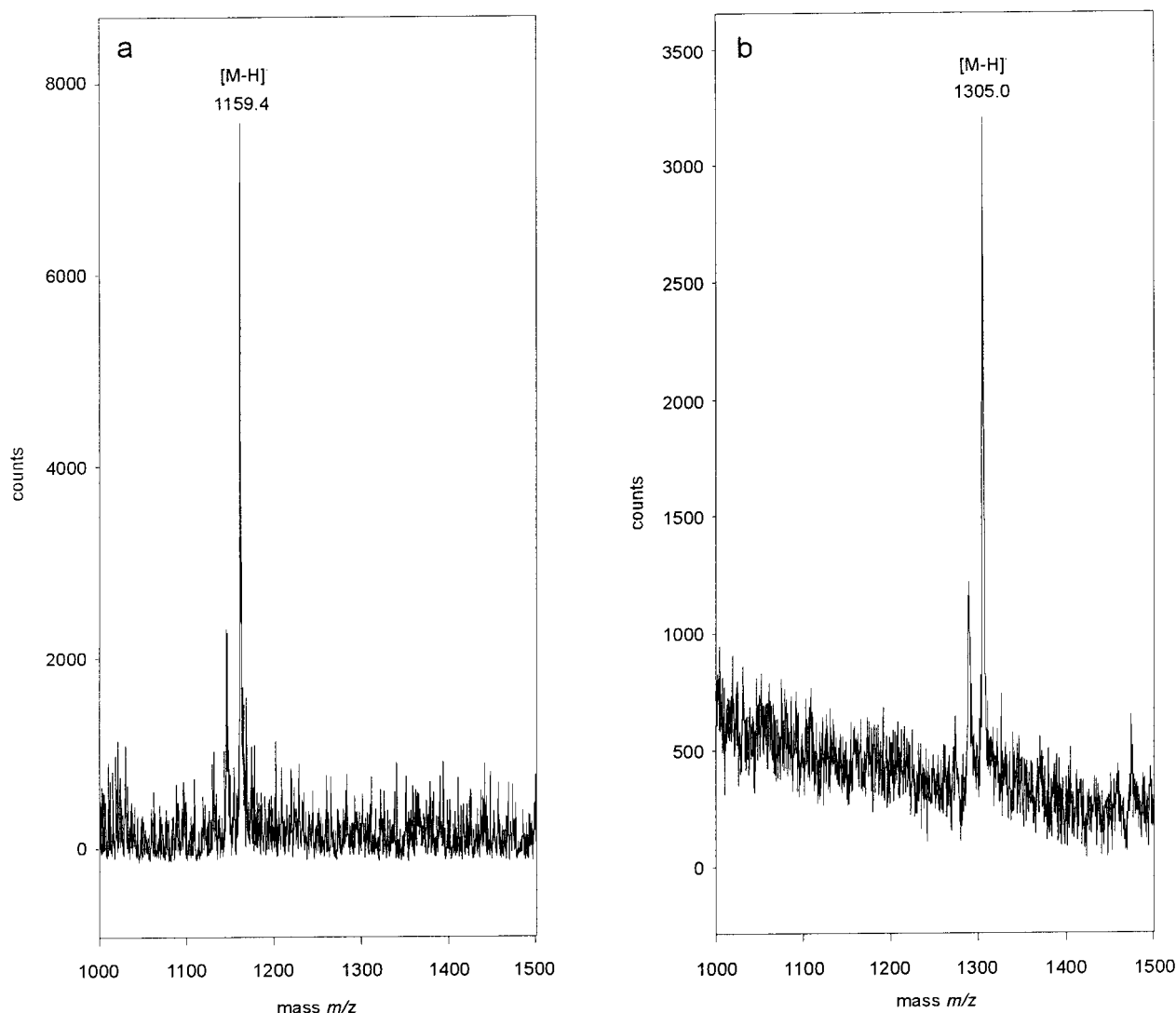


Figure 1. MALDI TOF mass spectra (negative-ion mode) of (a) Neu5AcHex₂HexNAc₂-pNp, and (b) Neu5AcHex₂dHexHexNAc₂-pNp.

β -1,4-linked Gal⁴ (D; pyranose ring form), respectively (compare with compounds **1–3** in Table 2 and literature data [39]). The three *N*-acetyl resonances representing three protons each, at δ 2.030, 2.003 and 1.899 could be attributed to Neu5Ac, GalNAc and GlcNAc, respectively [39]. The presence of only one Neu5Ac residue (E) in this oligosaccharide was confirmed by comparing the intensities of the H3e and H3a resonances at δ 2.753 and 1.790, respectively, with those of the discrete signals of the *p*-nitrophenyl moiety.

By means of 2D TOCSY most of the resonances present in the 1D spectrum could be identified (Table 2, compound **4**). In the TOCSY spectrum (100 ms, not shown) the anomeric track of the Gal⁴ residue D revealed three cross-peaks at δ 3.540 (H2), 4.097 (H3), and 3.951 (H4). When compared to the corresponding signals of precursor **2**, these resonances showed downfield shifts of 0.028 ppm for Gal⁴ H2, 0.447 ppm for Gal⁴ H3, and 0.033 ppm for Gal⁴ H4,

indicating that Neu5Ac is linked at O3 of Gal⁴. The anomeric track of the Gal³ residue B revealed three cross-peaks at δ 3.553 (H2), 3.654 (H3), and 3.928 (H4), in agreement with the terminal position of this residue (c.f. compound **2**, Table 2), thereby excluding a possible sialylation of the Gal³ residue. The ST3Gal3-side activity on core 1 galactose as observed by Kono *et al.* [40] was absent in our case. The combined MS and NMR results justify the conclusion that oligosaccharide **4** has the structure Neu5Ac (α 2-3) Gal(β 1-4)GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp).

The negative-ion mode MALDI TOF mass spectrum (Figure 1b) of compound **5** showed one major peak at m/z 1305.0 corresponding to the deprotonated pseudo-molecular ion of a hexasaccharide with the bruttoformula Neu5Ac Hex₂dHexHexNAc₂-pNp (calculated mass 1305.4).

The 1D ¹H NMR spectrum (Figure 2b) of compound **5** revealed five anomeric signals at δ 5.807 (³*J*_{1,2} 4.0 Hz), 5.030 (³*J*_{1,2} 4.0 Hz), 4.545 (³*J*_{1,2} 7.5 Hz), 4.482 (³*J*_{1,2} 7.5 Hz), and

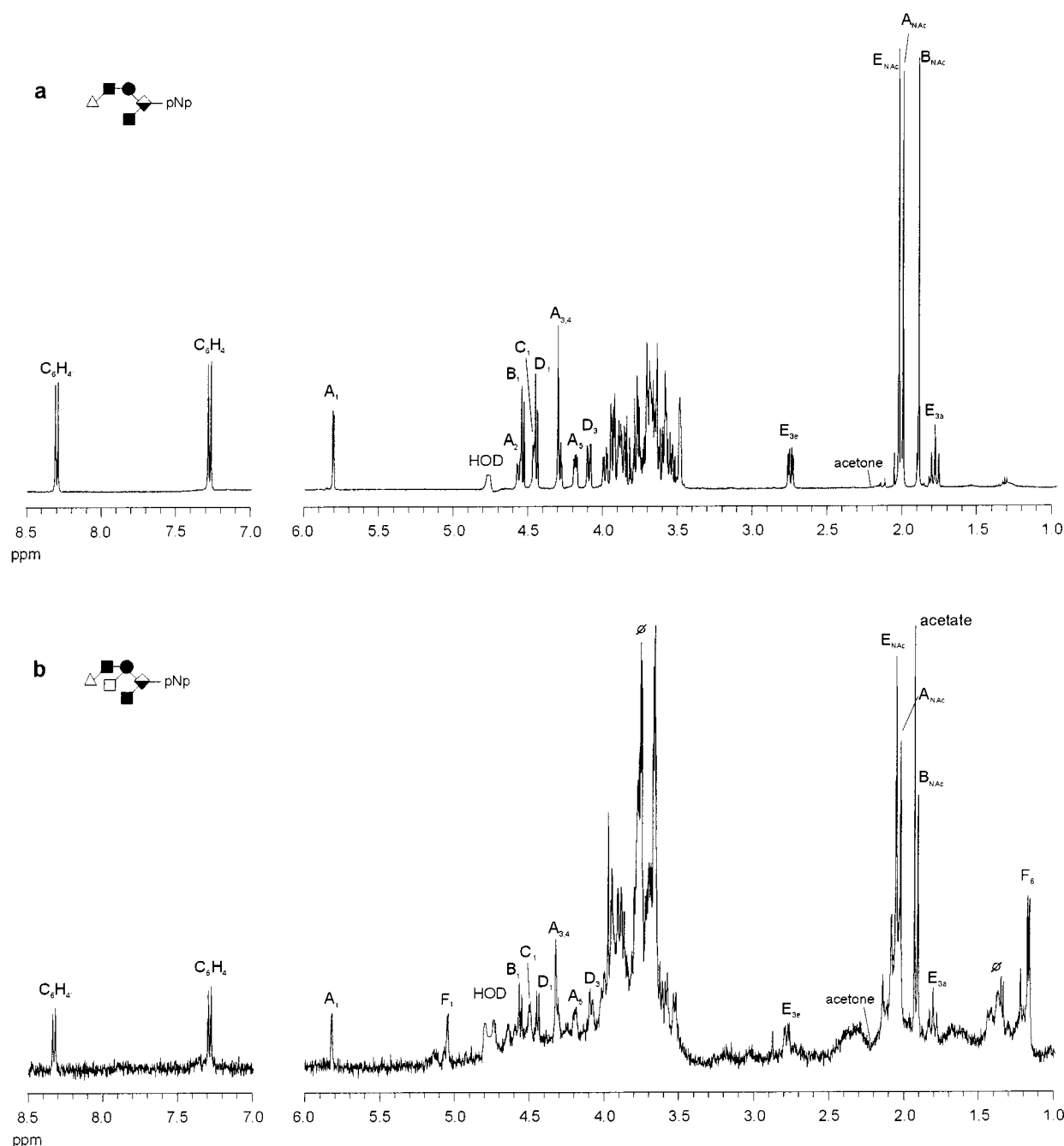
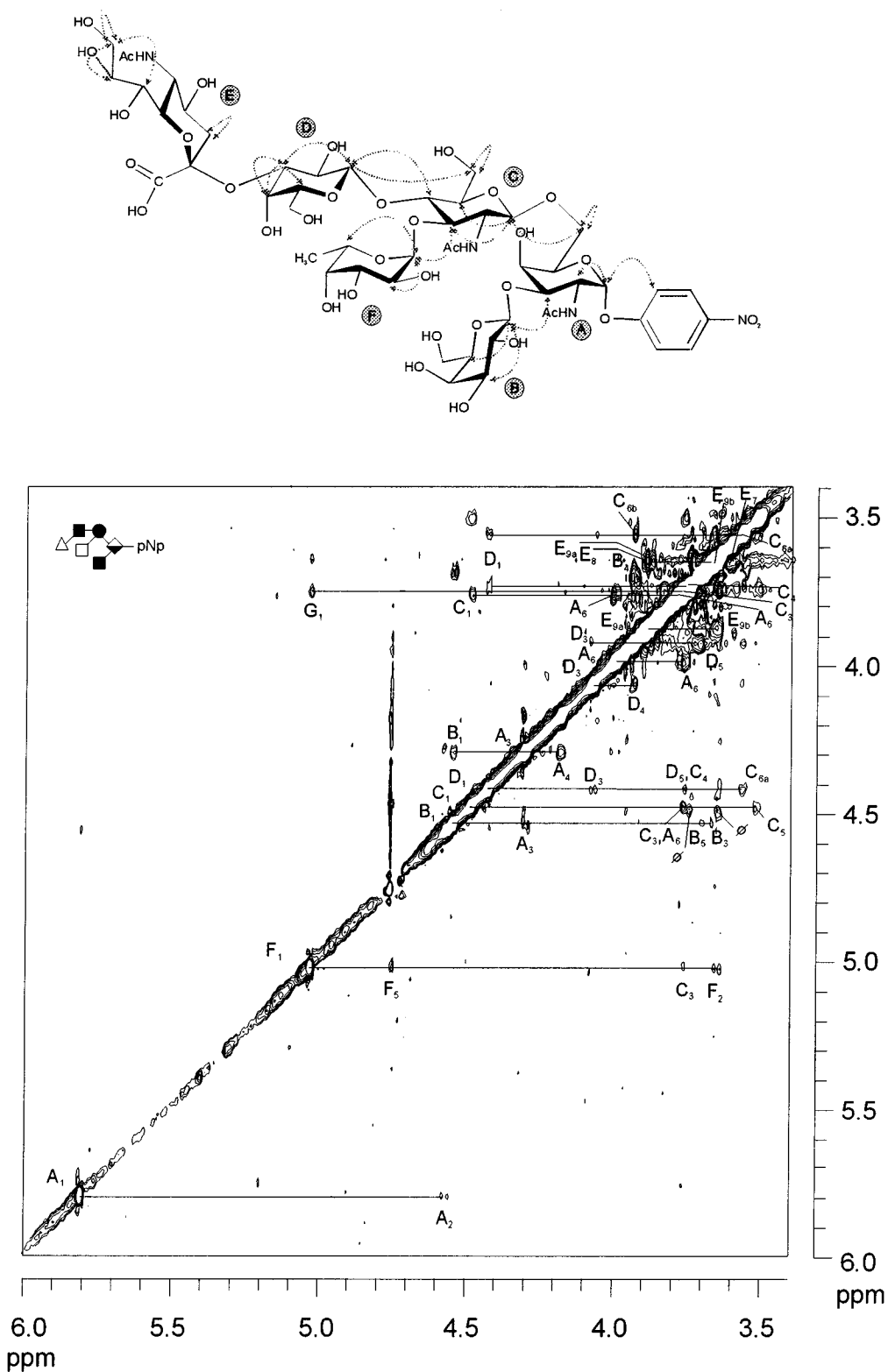


Figure 2. One-dimensional 500 MHz or 600 MHz ^1H NMR spectra of (a) Neu5Ac(α 2-3)Gal(β 1-4)GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp (**4**) (500 MHz) and (b) Neu5Ac(α 2-3)Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp (**5**) (600 MHz) in $^2\text{H}_2\text{O}$ at 300 K (referenced to internal acetone (δ 2.225)). Assignment in the Figure: A = GalNAc, B = Gal³, C = GlcNAc⁶, D = Gal^{4,6}, E = Neu5Ac, F = Fuc.

4.428 ($^3J_{1,2}$ 7.5 Hz), which could be identified on guidance of the NMR data of compounds **1–4** (Table 2). The additional anomeric resonance at δ 5.030 in compound **5** belonged to the incorporated α -Fuc residue (G; pyranose ring form). 2D TOCSY NMR spectroscopy allowed the identification of most of the signals (Table 2). The introduction of Fuc at O3 of GlcNAc (C) in **4** resulted in distinct downfield shifts of GlcNAc H1 (0.015 ppm), H2 (0.181 ppm), H3

(0.277 ppm), and H4 (0.082 ppm), a feature which is also observed when the NMR data of compounds **2** and **3** are compared (Table 2).

In order to confirm the various glycosidic linkages in compound **5** a 2D off-resonance ROESY (Figure 3) experiment was performed. The interresidual cross-peaks between Gal³ H1 and GalNAc H3 (δ 4.545/4.291), GlcNAc H1 and GalNAc H6a (δ 4.482/3.763), Fuc H1 and GlcNAc



H3 (δ 5.030/3.764), and between Gal⁴ H1 and GlcNAc H4 (δ 4.428/3.745) proved the presence of the Gal(β 1-3)GalNAc, GlcNAc(β 1-6)GalNAc, Fuc(α 1-3)GlcNAc, and Gal(β 1-4)GlcNAc linkages, respectively. It should be noted that the interresidual cross-peak between Neu5Ac H3e and Gal⁴ H3 as reported by Ball *et al.* [41] was not observed in our study. The combined MS and NMR data indicate that oligosaccharide **5** has the structure Neu5Ac(α 2-3)Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp).

Discussion

The production of selectin blockers seems to be a rewarding task with respect to several therapeutic targets, such as inflammatory diseases, transplant rejection and metastasis. This new class of anti-adhesion compounds can be enzymically generated by taking advantage of the high stereo- and regioselectivity of glycosyltransferases. This approach which ascertains an easy access to the naturally occurring PSGL-1 type sLe^x epitope, in relatively high yields, opens the possibility for the synthesis of glycopeptides by making use of the four cloned animal polypeptide GalNAc-transferases [42–46]. The structural identity with the natural ligand would not only render these compounds highly compatible in the competition with PSGL-1, but also assure a prolonged life-time and reduced antigenicity when compared with the non-natural ligands.

To demonstrate the suitability of glycosyltransferases for the synthesis of complex oligosaccharides, we used GalNAc(α 1-pNp as the starting substrate for the elongation of O-linked glycans. As shown previously [30] the branching C2GnT has a strict requirement for a 3-substitution of GalNAc. Even though a β 3-GalT, involved in the GM1/GD1 synthesis, transferring Gal to lipid-linked GalNAc [47], has been cloned, yet no recombinant enzyme is available elongating peptide-bound GalNAc. The rapid degradation of UDP-Gal in our system correlates well with the observation [48] that UDP-Gal decomposes rapidly in the presence of Mn²⁺, a metal ion needed to maintain β 3-GalT activity. Therefore, a two step incubation was carried out, resulting in a final yield of 52% for Gal(β 1-3)GalNAc(α 1-pNp (core 1 structure).

To date three different C2GnTs, of use for the branching of the core 1 structure, have been cloned [49–51]. Two of the three transferases [50,51] are also capable of synthesizing the core 4 structure (GlcNAc(β 1-6)[GlcNAc(β 1-3)]GalNAc). As shown recently, the core 1 disaccharide can alternatively be branched with the β -N-acetyl-D-hexosaminidase from *Nocardia orientalis* [52]; unfortunately, the yield is only around 6%. Applying a crude mouse kidney C2GnT preparation, the branching of a core 1 structure was realized in a yield of 74% [53]. By the use of a recombinant purified mouse C2GnT we achieved a yield of >85% for GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp (core 2 structure).

Recently, β -galactosidase from bovine testes was used in a one pot reaction together with a recombinant β -1,6-GlcNAc transferase. The galactosidase, which reversibly links galactose via a (β 1-3) linkage to N-acetylgalactosamine, provides the substrate for the GlcNAc transferase *in situ* [54]. Since this synthesis ended up with a mixture of core 2 and core 6 (GlcNAc(β 1-6)GalNAc) structures, and can only be driven towards high yields of the core 6 structure (yield >90%), we preferred the use of a crude β 3-GalT preparation in combination with C2GnT.

The core 2 structure can be galactosylated at O4 of GlcNAc by various β 4-GalTs. By now, a whole family of β 4-GalTs has been identified and cloned (reviewed in [55]). Although β 4-GalT1 has been reported to be inefficient for the elongation of the core 2 structure [56], the purified human milk enzyme, known as β 4-GalT1, was successfully applied to extend the core 2 structure, yielding Gal(β 1-4)GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp. In spite of the earlier reported data that a strong inhibition of β 4-GalT1 should exist for core 2 acceptor concentrations of 5 mM [53], we were able to reach a yield of >85% with 200 mU of enzyme at an acceptor concentration of 10 mM. It should be noted that within 5 h of incubation we did not achieve the earlier reported 100% yield with the enzyme used in [53].

The tetrasaccharide as generated above, was further extended by sialic acid using ST3Gal3. Out of the four to date cloned enzymes only ST3Gal3 and ST3Gal4 have been demonstrated to sialylate N-acetyllactosamine (LacNAc) [40]. In our studies we used purified recombinant rat ST3Gal3, despite the preference of this enzyme for Gal(β 1-3)GlcNAc (lacto-N-biose). Both ST3Gal3 and ST3Gal4 have a side activity in sialylating the Gal residue in a core 1 structure [40]. However, Gal(β 1-4)GlcNAc(β 1-6)[Neu5Ac(α 2-3)Gal(β 1-3)]GalNAc(α 1-pNp was not found in our incubation mixture. This indicates that the side activity is strictly confined to the absence of a Gal(β 1-4)X and the presence of a Gal(β 1-3)X acceptor. Using a similar substrate in an earlier study, a yield of 68% was obtained with the partially purified recombinant rat ST3Gal3 [53]. Here, we obtained Neu5Ac(α 2-3)Gal(β 1-4)GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp in a yield of 87% via a two step incubation using 170 and 72 mU of the purified enzyme.

To obtain the sLe^x epitope, the foregoing structure was fucosylated at O3 of GlcNAc. At present five cloned α 3-FucTs (α 3-FucT3 - α 3-FucT7), displaying different acceptor specificities, are known. α 3-FucT4 fucosylates nearly exclusively LacNAc, whereas α 3-FucT7 only acts on (α 2-3)-sialylated LacNAc [57,58]. α 3-FucT6, used in this study, fucosylates both sialylated and non-sialylated LacNAc. This enzyme, which has been shown to be highly active *in vivo* [59], is also of interest for the preparation of difucosyl sLe^x structures on poly-LacNAc chains [60]. The expression system for α 3-FucT6, used in our study, prevails over the system

described by Licari *et al.* [38], since no exoglycosidases are present in the supernatant which cause degradation of the acceptor substrate. In a 6 h incubation with 70 mU of recombinant α 3-FucT6, Neu5Ac(α 2-3)Gal(β 1-4)[Fuc(α 1-3)]GlcNAc(β 1-6)[Gal(β 1-3)]GalNAc(α 1-pNp) was synthesized in 100% yield.

In summary, we performed the first full enzymic synthesis of a core 2 type sLe^x-containing hexasaccharide in an overall yield of about 32%. Once recombinant β 3-GalT forming the core 1 structure will become available, scaling-up of the procedure described here should be feasible.

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References

- Walz G, Aruffo A, Kolanus W, Bevilacqua M, Seed B (1990) *Science* **250**: 1132–35.
- Foxall C, Watson SR, Dowbenko D, Fennie C, Lasky LA, Kiso M, Hasegawa A, Asa D, Brandley BK (1992) *J Cell Biol* **117**: 895–902.
- Kim YJ, Borsig L, Varki NM, Varki A (1998) *Proc Natl Acad Sci USA* **95**: 9325–30.
- Yamaguchi A, Ding K, Maehara M, Goi T, Nakagawara G (1998) *Oncology* **55**: 357–62.
- Varki A (1997) *J Clin Invest* **99**: 158–62.
- Kansas GS (1996) *Blood* **88**: 3259–87.
- Turunen JP, Paavonen T, Majuri ML, Tiisala S, Mattila P, Mennander A, Gahmberg CG, Hayry P, Tamatani T, Miyasaka M, Renkonen R (1994) *Eur J Immunol* **24**: 1130–36.
- McEver RP, Moore KL, Cummings RD (1995) *J Biol Chem* **270**: 11025–28.
- Han KT, Sharar SR, Phillips ML, Harlan JM, Winn RK (1995) *J Immunol* **155**: 4011–15.
- Mulligan MS, Paulson JC, DeFrees S, Zheng ZL, Lowe JB, Ward PA (1993) *Nature* **364**: 149–51.
- Buerke M, Weyrich AS, Zheng Z, Gaeta FC, Forrest MJ, Lefer AM (1994) *J Clin Invest* **93**: 1140–48.
- Murohara T, Margiotta J, Phillips LM, Paulson JC, DeFrees S, Zalipsky S, Guo LS, Lefer AM (1995) *Cardiovasc Res* **30**: 965–74.
- Nguyen M, Eilber FR, DeFrees S (1996) *Biochem Biophys Res Commun* **228**: 716–23.
- Kameyama A, Ishida H, Kiso M, Hasegawa A (1991) *Carbohydr Res* **209**: C1–C4.
- Kiyoi T, Nakai Y, Kondo H, Ishida H, Kiso M, Hasegawa A (1996) *Bioorg Med Chem* **4**: 1167–76.
- Baba K, Iwata N, Hamajima H, Ikami T, Ishida H, Hasegawa A, Kiso M (1998) *Biosci Biotechnol Biochem* **62**: 590–92.
- Lin CH, Shimazaki M, Wong C-H, Koketsu M, Juneja LR, Kim M (1995) *Bioorg Med Chem* **3**: 1625–30.
- Ichikawa Y, Lin YC, Dumas DP, Shen GJ, Garcia-Junceda E, Williams MA, Bayer R, Kretscham C, Walker LE, Paulson JC, Wong C-H (1992) *J Am Chem Soc* **114**: 9283–98.
- Nelson RM, Cecconi O, Roberts WG, Aruffo A, Linhardt RJ, Bevilacqua MP (1993) *Blood* **82**: 3253–58.
- Mulligan MS, Polley MJ, Bayer RJ, Nunn MF, Paulson JC, Ward PA (1992) *J Clin Invest* **90**: 1600–07.
- Polley MJ, Phillips ML, Wayner E, Nudelman E, Singhal AK, Hakomori S, Paulson JC (1991) *Proc Acad Natl Sci USA* **88**: 6224–28.
- Renkonen O, Toppila S, Penttilä L, Salminen H, Helin J, Maaheimo H, Costello CE, Turunen JP, Renkonen R (1997) *Glycobiology* **7**: 453–61.
- Miyauchi H, Yuri M, Tanaka M, Kawamura N, Hayashi M (1997) *Bioorg Med Chem Lett* **7**: 989–92.
- Simanek EE, McGarvey GJ, Jablonowski JA, Wong C-H (1998) *Chem Rev* **98**: 833–62.
- Hiruma K, Kajimoto T, Weitz-Schmidt G, Ollmann I, Wong C-H (1996) *J Am Chem Soc* **118**: 9265–70.
- Wittmann V, Takayama S, Gong KW, Weitz-Schmidt G, Wong C-H (1998) *J Org Chem* **63**: 5137–43.
- Liu W, Ramachandran V, Kang J, Kishimoto TK, Cummings RD, McEver RP (1998) *J Biol Chem* **273**: 7078–87.
- Brockhausen I, Moller G, Pollex-Kruger A, Rutz V, Paulsen JC, Matta KL (1992) *Biochem Cell Biol* **70**: 99–108.
- Bendiak B, Schachter H (1987) *J Biol Chem* **262**: 5775–83.
- Zeng S, Dinter A, Eisenkrätzer D, Biselli M, Wandrey C, Berger EG (1997) *Biochem Biophys Res Commun* **237**: 653–58.
- Gerber AC, Kozdrowski I, Wyss SR, Berger EG (1979) *Eur J Biochem* **93**: 453–60.
- Hård K, van Zadelhoff G, Moonen P, Kamerling JP, Vliegenthart JFG (1992) *Eur J Biochem* **209**: 895–915.
- Bax A, Davis J (1985) *J Magn Reson* **65**: 355–60.
- Bax A, Davis J (1985) *J Magn Reson* **63**: 207–13.
- Desvaux H, Berthault P, Birlirakis N, Goldman M, Piotto M (1995) *J Magn Reson* **113**: 47–52.
- Unverzagt C, Kunz H, Paulson JC (1990) *J Am Chem Soc* **112**: 9308–09.
- Tsuji S, Datta AK, Paulson JC (1996) *Glycobiology* **6**: R5–7.
- Licari PJ, Jarvis DL, Bailey JE (1993) *Biotechnol Prog* **9**: 146–52.
- Pollex-Kruger A, Meyer B, Stuike-Prill R, Sinnwell V, Matta KL, Brockhausen I (1993) *Glycoconjugate J* **10**: 365–80.
- Kono M, Ohyama Y, Lee YC, Hamamoto T, Kojima N, Tsuji S (1997) *Glycobiology* **7**: 469–79.
- Ball GE, O'Neill RA, Schultz JE, Lowe JB, Weston BW, Nagy JO, Brown EG, Hobbs CJ, Bednarski MD (1992) *J Am Chem Soc* **114**: 5449–51.
- Clausen H, Bennett EP (1996) *Glycobiology* **6**: 635–46.
- Homa FL, Hollander T, Lehman DJ, Thomsen DR, Elhammer AP (1993) *J Biol Chem* **268**: 12609–16.
- White T, Bennett EP, Takio K, Sorensen T, Bonding N, Clausen H (1995) *J Biol Chem* **270**: 24156–65.
- Bennett EP, Hassan H, Clausen H (1996) *J Biol Chem* **271**: 17006–12.
- Hagen FK, Ten Hagen KG, Beres TM, Balys MM, VanWuyckhuyse BC, Tabak LA (1997) *J Biol Chem* **272**: 13843–48.
- Amado M, Almeida R, Carneiro F, Lavery SB, Holmes EH, Nomoto M, Hollingsworth MA, Hassan H, Schwientek T, Nielsen PA, Bennett EP, Clausen H (1998) *J Biol Chem* **273**: 12770–78.

- 48 Nunez HA, Barker R (1976) *Biochemistry* **15**: 3843–47.
- 49 Bierhuizen MFA, Fukuda M (1992) *Proc Natl Acad Sci USA* **89**: 9326–30.
- 50 Yeh JC, Ong E, Fukuda M (1999) *J Biol Chem* **274**: 3215–21.
- 51 Schwientek T, Nomoto M, Levery SB, Merkx G, van Kessel AG, Bennett EP, Hollingsworth MA, Clausen H (1999) *J Biol Chem* **274**: 4504–12.
- 52 Murata T, Itoh T, Usui T (1998) *Glycoconjugate J* **15**: 575–82.
- 53 Oehrlein R, Hindsgaul O, Palcic MM (1993) *Carbohydr Res* **244**: 149–59.
- 54 Dudziak G, Zeng S, Berger EG, Gutiérrez Gallego R, Kamerling JP, Kragl U, Wandrey C (1998) *Bioorg Med Chem Lett* **8**: 2595–98.
- 55 Breton C, Bettler E, Joziassé DH, Geremia RA, Imberty A (1998) *J Biochem* **123**: 1000–09.
- 56 Ujita M, McAuliffe J, Schwientek T, Almeida R, Hindsgaul O, Clausen H, Fukuda M (1998) *J Biol Chem* **273**: 34843–49.
- 57 Niemelä R, Natunen J, Majuri M-L, Maaheimo H, Helin J, Lowe JB, Renkonen O, Renkonen R (1998) *J Biol Chem* **273**: 4021–26.
- 58 Grabenhorst E, Nimtz M, Costa J, Conradt HS (1998) *J Biol Chem* **273**: 30985–94.
- 59 Kimura H, Shinya N, Nishihara S, Kaneko M, Irimura T, Narimatsu H (1997) *Biochem Biophys Res Commun* **237**: 131–37.
- 60 Weston BW, Smith PL, Kelly RJ, Lowe JB (1992) *J Biol Chem* **267**: 24575–84.

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